

Evidence for Arbovirus Dissemination Conduits from the Mosquito (Diptera: Culicidae) Midgut

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ABSTRACT The mechanism by which arboviruses bypass the basal lamina of mosquito midgut cells and enter the body cavity has been unclear. Experiments using Venezuelan equine encephalitis viral replicon particles, which express the green fluorescent protein gene in cells, indicate the operation of tissue conduits, possibly involving tracheae and visceral muscles, that facilitate virus movement through the basal lamina. Ultrastructural studies of the midgut reveal evidence for possible complete penetration of the basal lamina by tracheal cells and regions of modified basal lamina associated with visceral muscle. The modified basal lamina closely resembles proventricular matrix material known to allow virus passage.

KEY WORDS arbovirus, mosquito, midgut, basal lamina, tracheae

ARBOVIRUSES ENTER AND EXIT from their mosquito hosts in association with blood feeding (Chamberlain and Sudia 1961, Hardy 1988). The overall sequence is as follows: 1) a female mosquito feeds on a viremic vertebrate host; 2) viruses in the blood meal infect midgut epithelial cells, replicate, and subsequently escape from the midgut (the process of dissemination); 3) viruses infect tissues and organs, including the salivary glands, within the body cavity (hemocoel); and 4) during the ingestion of a subsequent blood meal, viruses, along with saliva, are injected into a new vertebrate host. In some cases, arboviruses infect developing eggs and are transmitted transovarially to the next generation (Turell 1988). To be transmitted to a vertebrate host or a subsequent mosquito generation, an arbovirus must be able to reach and infect the salivary glands or the ovaries, respectively. Studies of arboviral pathogenesis in mosquitoes are essential to understand their horizontal and vertical vector competence for arboviruses (Hardy 1988, Leake 1992). For several years, we have studied the pathogenesis of Venezuelan equine encephalitis (VEE, *Alphavirus*, *Togaviridae*) and Rift Valley fever (RVF, *Phlebovirus*, *Bunyaviridae*) viruses in the mosquitoes *Ochlerotatus taeniorhynchus* (Wiedemann) and *Culex pipiens* L., respectively. VEE virus has a single-stranded RNA genome with a diameter of 60 nm and can cause serious illness in vertebrates, particularly horses and humans (Walton and Grayson 1988). *Oc. taeniorhyn-*

chus is recognized as a potential vector of this virus (Turell et al. 1992). RVF virus has a tripartite, single-stranded RNA genome and ranges in diameter from 90 to 120 nm (Meegan and Bailey 1988). Rift valley fever is enzootic and sporadically epidemic in sub-Saharan Africa, but since the late 1970s, several epidemics have occurred north of the Sahara, posing a serious health threat to humans and livestock (Meegan 1979, Meegan et al. 1980, Abd.el-Rahim et al. 1999, Ahmad 2000).

Studies of the mosquito midgut basal lamina have revealed pore sizes significantly smaller than arboviruses (Hardy 1988, Reddy and Locke 1990). Therefore, a key issue in arboviral pathogenesis in mosquitoes is how viruses bypass the noncellular basal lamina that coats the epithelia, including those of the midgut and salivary glands. A potential route of dissemination of viruses from the midgut epithelium and into the body cavity is via tracheal cells. These cells are formed into a series of continuous tubular channels (tracheae), which communicate with the external environment via openings (spiracles) in the integument and are responsible for transporting oxygen to the various tissues (Romoser and Stoffolano 1998). Spiracles open into large tracheae that branch and become progressively smaller, ultimately ending in a single cell (tracheoblast) that contains one or more open channels, tracheoles, contiguous with the lumen of the tracheae. Oxygen enters tissues at the tracheoblast level. Tracheae arise as ectodermal invaginations and as such retain a cuticular lining or intima, which is typically in the form of helical folds. Midgut tracheae are associated intimately with visceral muscle fibers that are arranged in a more or less regular network of circular and longitudinal fibers (Fig. 1A). Visceral muscle fibers are invested in an ectod (outer) layer of basal lamina that seems to separate them

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completely from the hemocoel (Fig. 5A). Midgut tracheae, which are contiguous with the rest of the tracheal system, penetrate this outer layer. Earlier studies of mosquito midgut ultrastructure did not report complete tracheal penetration of the basal lamina (Hecker et al. 1971, Hecker 1977).

The first part of our research uses VEE-viral replicon particles (VRPs), which express green fluorescent protein (GFP) in infected cells. VEE-VRPs have envelopes that are identical to fully infectious VEE virus but are genetically modified and only capable of a single round of infection and expression of GFP (Pushko et al. 1997; Schlesinger and Dubensky 1999; MacDonald and Johnston 2000; Schlesinger 2000, 2001; Lundstrom 2002; Rayner et al. 2002). With these characteristics, VEE-VRPs have enabled us to identify the first cells and tissues infected when they are injected into the mosquito hemocoel or introduced orally with a blood meal. For the second part of this study, we examined electron micrographs of the midgut of *Cx. pipiens* prepared for studies of RVF virus, and histological preparations of *Cx. pipiens* and *Oc. taeniorhynchus*.

Materials and Methods

Infection, Incubation, and Dissection of Mosquitoes. Female *Oc. taeniorhynchus* (MAVEL strain) were intrahemocoelically infected (via the thorax, intrathoracic, "IT-inoculation") with VRPs derived from the V3000 clone of wild-type Trinidad Donkey (Davis et al. 1995) or 3526 (Davis et al. 1989) strains of VEE virus and incubated for various periods. Approximately 0.5 μ l of VEE-VRP preparation containing $\approx 3 \times 10^3$ fluorescence-forming units (FFUs; see below) was injected into each mosquito. Controls were injected with Hanks' balanced salts solution (HBSS) or fed uninfected blood and incubated for equivalent periods. Dissections of both fresh and fixed specimens (10% buffered paraformaldehyde) were prepared and examined, using epifluorescence microscopy. Dissections were carried out in HBSS, distilled water or fixative in a deep well slide, using a Bausch & Lomb dissecting microscope. Dissected organs and tissues of VEE/VRP-injected and HBSS-injected specimens were placed in individual wells of Teflon-coated, 10-well slides. A coverslip was applied to each completed slide using a 3:1 solution of glycerin/HBSS as a mounting medium and studied using a Zeiss Axioplan or a Nikon Eclipse E600 epifluorescence microscope. The majority of VEE/VRP-infected specimens displayed GFP-positive cells, whereas none of the control (HBSS-injected) specimens displayed GFP-positive cells. Autofluorescence was observed in scattered cells among the fat body and in cells associated with the rectal papillae. Tissues with cuticular elements, i.e., tracheae and facets of the compound eyes, sometimes produced a nonspecific amber glow that was readily distinguishable from GFP expression. Results were identical in both fresh and fixed specimens.

To introduce VEE/VRP-infected blood into the mosquito midgut, ≈ 8 -d-old adult *Oc. taeniorhynchus*

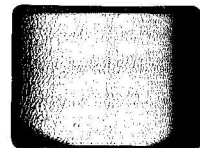
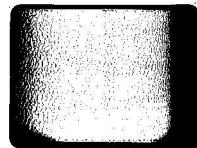
that had been denied access to sugar solution for ≈ 24 h were provided with heparinized goose blood via an artificial membrane feeder using Baudrausch membranes. The experimental group received a 1:10 dilution of V3000-VRPs in goose blood from a stock solution containing $\approx 1 \times 10^7$ FFUs/ml. The control group received only goose blood. Specimens were dissected after 24, 48, 72, 102, and 104 h post-blood meal.

To test the possibility that heparin might influence virus-vector interactions, pre- and post-blood meal, and 14-d postinfection viral titers were compared in mosquitoes artificially fed with heparinized blood versus glass bead defibrinated blood. No significant differences were detected between any of the groups (unpublished data).

Production of Viral Replicon. To produce VEE-VRP particles (Pushko et al. 1997), an exogenous gene that codes for GFP is substituted for the genes that encode for the capsid and two envelope glycoproteins. The VEE virus RNA promoter sequence and the genes that encode for the viral replicase are left intact. The GFP gene along with the remainder of the VEE genome constitutes a "replicon." When this modified genome is introduced into cultured cells, the exogenous gene for GFP is expressed. When helper mRNAs, which code for VEE virus capsid and envelope glycoproteins, are introduced into cells along with the replicons, VEE/VRPs are produced. The envelope, with its two glycoproteins, is identical to that of an unmodified VEE virion, thereby enabling the VRPs to infect cells in a manner identical to VEE virus. Once in a host cell, VRPs are incapable of replicating, but a large amount of GFP is produced and infected cells are detectable with epifluorescence microscopy.

Titration of Fluorescence-Forming Units. VEE-VRPs expressing GFP were titrated by a method similar to plaque assay, except that fluorescing cells were counted instead of plaques. Baby hamster kidney cells were grown in eight-well Lab-Tek chambered slides (Nalge Nunc International, Naperville, IL) in minimal essential medium (MEM) supplemented with Earle's salts, 10% fetal bovine serum, 200 U/ml penicillin, 200 μ g/ml streptomycin, and 10 μ g/ml gentamicin sulfate until just subconfluent. Ten-fold serial dilutions of samples were made in supplemented MEM. The cell culture medium was removed from the slides, and duplicate wells were inoculated with 400 μ l of each sample dilution. After all the wells were inoculated with the appropriate dilutions, the slides were held at 37°C under 5% CO₂. After incubation for 24–28 h, cell culture chambers were removed from the slides. The slides were rinsed with phosphate-buffered saline (PBS), and mounted with PBS and coverslips. Wells were examined by epifluorescence microscopy from lowest to highest dilution. The number of fluorescing cells in wells containing 20–200 fluorescing cells was counted. The number of FFUs per milliliter was calculated by multiplying the average number of fluorescing cells per well by the dilution factor and by 2.5 to correct for the inoculum volume.

Electron Microscopy. Electron photomicrographs produced for earlier studies of RVF virus were reex-



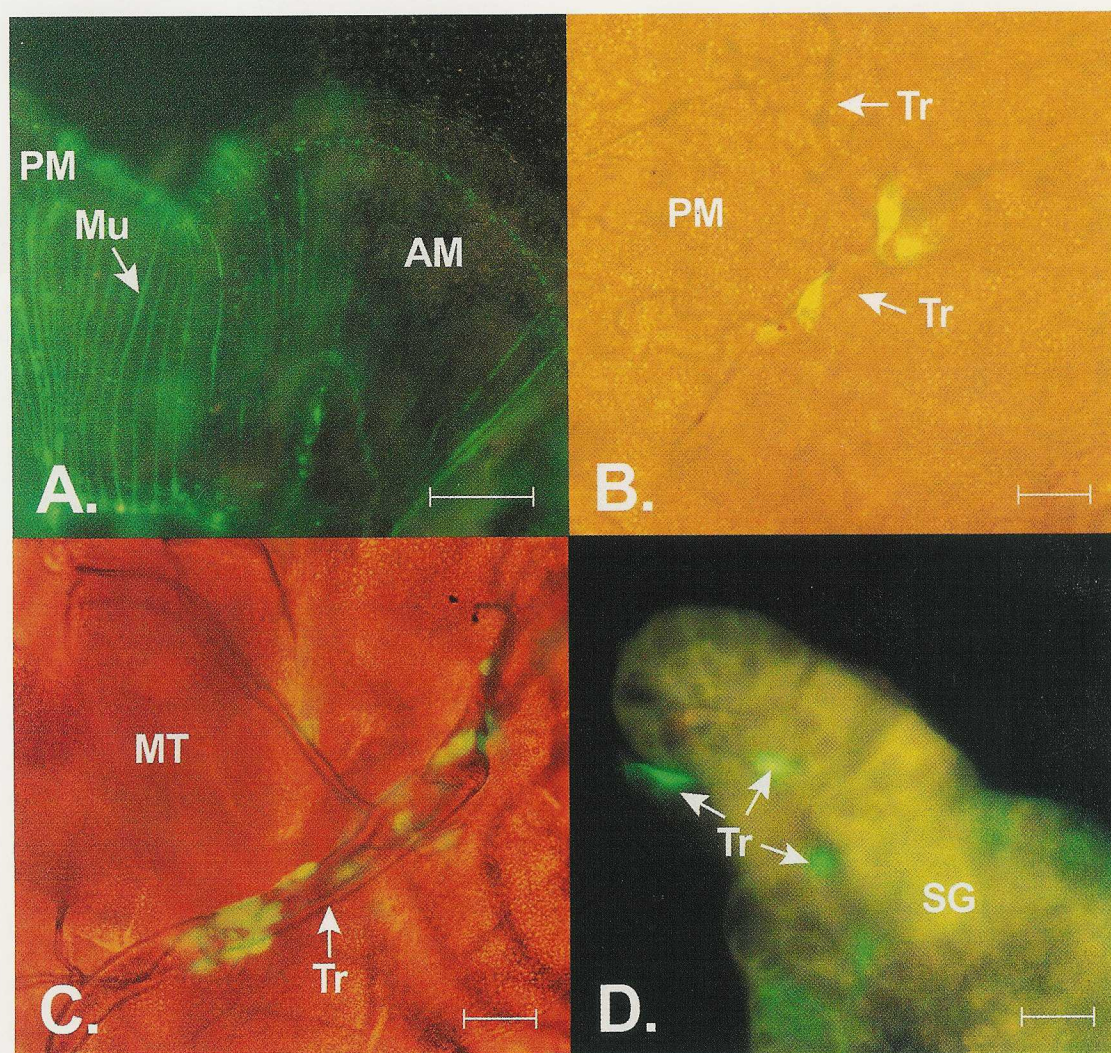


Fig. 1. VEE/VRP-infected midgut cells in *Oc. taeniorhynchus*. (A) GFP-positive visceral muscles at anterior-posterior midgut junction (IT-infected). (B) GFP-positive trachea on midgut (IT-infected). (C) GFP-positive tracheal cells associated with the posterior midgut and Malpighian tubules. (D) GFP-positive trachea associated with salivary gland. AM, anterior midgut; MT, Malpighian tubules; Mu, muscle; PM, posterior midgut; SG, salivary gland; Tr, trachea. A, scale bar, 15 μ m; B, scale bar, 25 μ m in B-D.

amined to test the hypothesis that tracheal cells penetrate the midgut basal lamina and have an intimate association within the intercellular spaces among the midgut epithelial cells. A description of the RVF virus strain, infection of the mosquitoes, and the techniques applied to produce and process ultrathin sections for electron microscopy can be found in Lerdthusnee et al. (1995).

Results and Discussion

Among the cells/tissues in mosquitoes infected after IT-inneculation of VEE-VRPs were those associated with the surfaces of the ventral chain ganglia and connecting nerves (14/22, 63.3% GFP-positive); midgut muscles (Fig. 1A; 15/20, 68.3% GFP-positive); cells

associated with the intussuscepted foregut portion of the proventriculus (12/22, 54.6% GFP-positive); and tracheal cells (17/22, 77.3% GFP-positive) associated with the alimentary canal (Fig. 1B and C), the salivary glands (Fig. 1D), and the ovaries. Tracheal and visceral muscle cells were consistently infected and to a greater extent than other tissues, whereas the midgut, and possibly the salivary gland epithelia, remained uninfected. In contrast, when fully infectious VEE virus is injected into the hemocoel, and its progress followed using nucleic acid hybridization and immunocytochemistry, midgut epithelial cells and salivary glands do become infected (Leon 1999). Mosquitoes orally exposed to VEE-VRPs in heparinized goose blood produced the following results: one or more GFP-positive midgut cells were found in 11/27

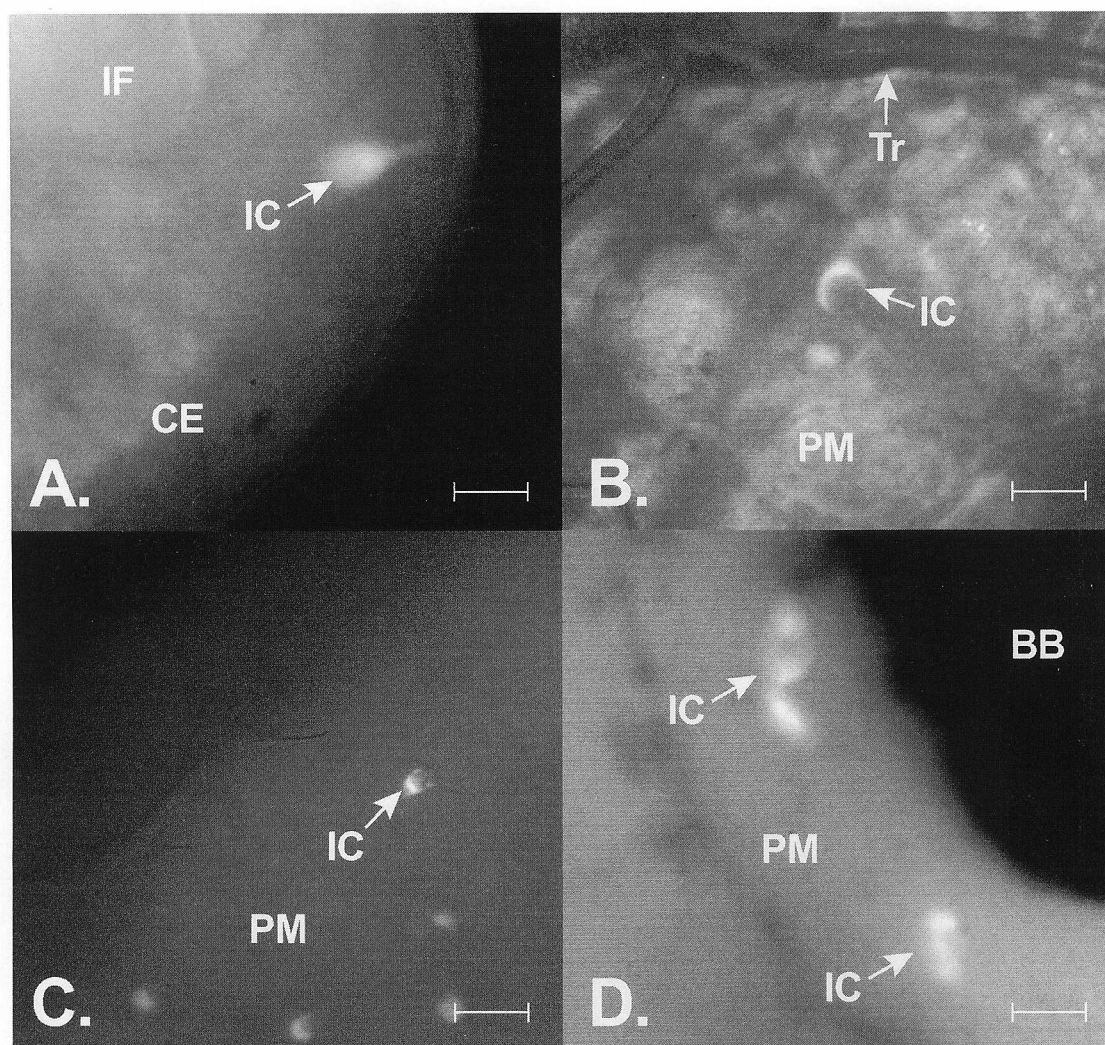


Fig. 2. VEE/VRP-infected midgut epithelial cells in orally infected *Oc. taeniorhynchus*. (A) epithelial cell in cardial epithelium, 102 h post-blood meal. (B–D) Posterior midgut epithelium; (B) 102 h post-blood meal; (C) 144 h post-blood meal, the unlabeled faint spots are also GFP positive; (D) 144 h post-blood meal, the epithelium has rolled back from the blood bolus. BB, blood bolus; CE, cardial epithelium; IC, infected cell; IF, intussuscepted foregut; PM, posterior midgut; Tr, trachea. Scale bar, 20 μ m.

(40.7%) of dissections (2/6 in 24-h group; 2/8 in 48-h; 1/4 in 72-h; 3/4 in 102-h; 3/5 in 144-h). None of the 12 negative controls, 1–5 of which were dissected in association with each incubation time, contained GFP-positive cells. Based on these results, it is clear that midgut epithelial cells can be infected by VRPs (Fig. 2A–D) and that midgut cells are the only cells infected by the oral route.

Summarizing to this point: 1) VEE-VRPs and VEE virions have identical envelopes and envelope glycoproteins and hence are capable of infecting the same cell types; 2) VEE-VRPs do not replicate and hence have enabled us to identify the first cells infected when they are introduced by a given route; 3) VRPs introduced into the hemocoel infect only midgut mus-

cles and tracheae, but not midgut epithelial cells; 4) VEE virus introduced into the hemocoel does not infect midgut cells; and 5) when introduced orally, both viable virus and VRPs infect midgut cells. In view of these facts, we conclude that viable VEE virus introduced into the hemocoel must infect the complex of tracheae and muscles before it infects midgut epithelial cells. Viable virus, therefore, must infect the midgut indirectly via a "conduit," and muscles and tracheae seem to be involved. Because the basal lamina lies between the midgut epithelium and the hemocoel; and has pore sizes much smaller than VRPs or virions, it is logical to conclude that it is the barrier that prevents midgut epithelial infection with VRPs. Furthermore, for the same reason, it is logical to assume

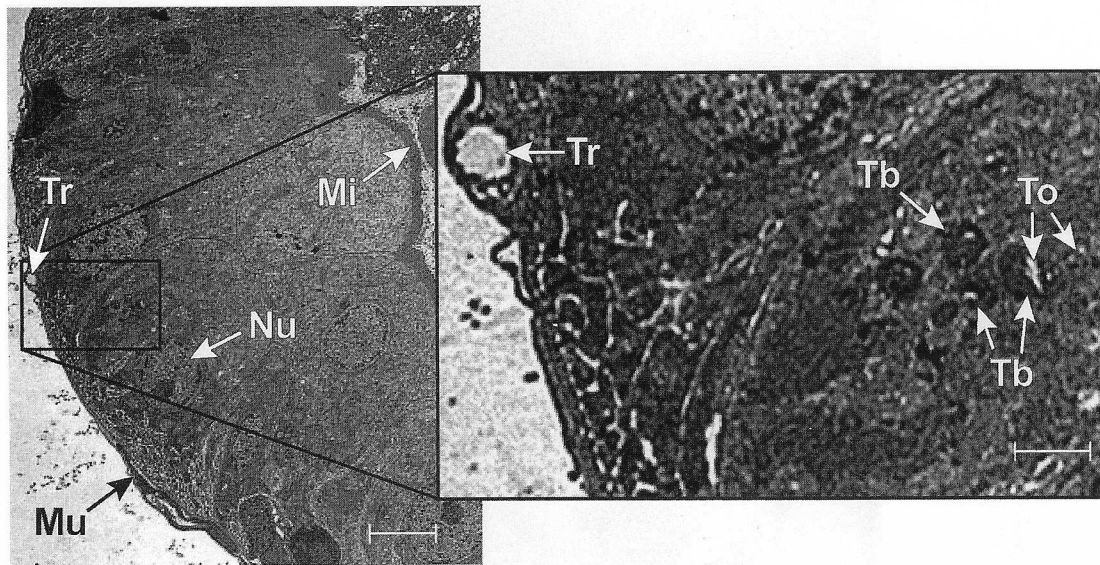


Fig. 3. Electron micrograph of sagittal section of cardinal epithelium of *Cx. pipiens*. Inset shows putative tracheal cells and tracheoles. Mi, microvilli; Mu, muscle; Nu, nucleus; Tb, tracheoblast; To, tracheole; Tr, trachea. Scale bar, original, 4 μ m; enlarged, 1 μ m.

that the basal lamina acts as a barrier to virus passage in the reverse direction, that is, from the midgut epithelium into the hemocoel.

Based on the results of the VEE-VRP study, we examined the association between the midgut epithelium, tracheae, and visceral muscles. There is evidence that the baculoviruses use a tracheal route as they spread within their insect hosts (Engelhard et al. 1994, Kirkpatrick et al. 1994, Barrett et al. 1998). Engelhard et al. (1994) studied the progression of a nuclear polyhedrosis virus infecting larval *Trichoplusia ni* (Hübner) and found that the earliest evidence of infection beyond the midgut was in tracheoblasts and tracheal epidermal cells associated with foci of infection in the midgut epithelium. In light of the work of Locke (1985, 1986), they postulated that rapid spread of virus in the hemocoel could occur via intercellular spaces associated with tracheal epithelia. Furthermore, and directly pertinent to arboviruses, Bowers et al. (1995) demonstrated the presence and persistence of Sindbis virus in tracheal cells of infected mosquitoes. Chandler et al. (1998) found evidence of ovarian infection with LaCrosse virus before dissemination of the virus from the midgut and suggested the possible transport of virus directly from the midgut to the ovaries via tracheal cells. Examination of electron micrographs of the midgut epithelium in *Cx. pipiens* revealed evidence of a possible intimate association between tracheae/tracheoles and midgut epithelial cells. In Fig. 3 putative tracheal cells seem to penetrate deeply into the midgut (cardial) epithelium. Complete tracheal penetration of the basal lamina in mosquitoes would be consistent with ultrastructural studies of the midgut in other kinds of insects in which tracheal cells in direct plasma membrane have been described in con-

tact with midgut epithelial cells (Maina 1989, Volkman 1997). It has been speculated that insect viruses (Engelhard et al. 1994) and the *Alphavirus* Sindbis could use the tracheal system as an "efficient vehicle" (Bowers et al. 1995) for the spread of virus beyond the midgut, which is supported by our preliminary ultrastructural evidence (Fig. 4).

Ultrastructural examination of the association between visceral muscles and the midgut epithelium reveals another plausible route of viral egress from midgut epithelial cells. In areas of very close contact between visceral muscle fibers and the midgut epithelium (Fig. 5A and B), the basal lamina commonly looks very different than in locations where there are no close muscle fibers. Typically, the basal lamina is composed of regular, apparently closely packed, lamellae, but in proximity to the entod (inner) surface of a muscle fiber, the basal lamina commonly seems porous or "spongy" and closely resembles the matrix material found in the proventriculus (Fig. 5C). RVF virions have been shown to penetrate this matrix material in the proventriculus (Lerdthusnee et al. 1995; Fig. 5C), indicating that they might likewise be able to penetrate the modified basal lamina in areas of close contact between visceral muscle and the midgut epithelium. Lerdthusnee et al. (1995) demonstrated that RVF virions bud into the basal labyrinth, a reticulate network of intercellular spaces located basally in midgut epithelial cells (Fig. 5D). It is clear that the spaces of the basal labyrinth are contiguous with the entod surface of the basal lamina (Fig. 5A, B, and D). Therefore, arboviruses that have budded into the basal labyrinth in the vicinity of modified basal lamina or have reached such a location by moving through the basal labyrinth, or perhaps have budded directly into the

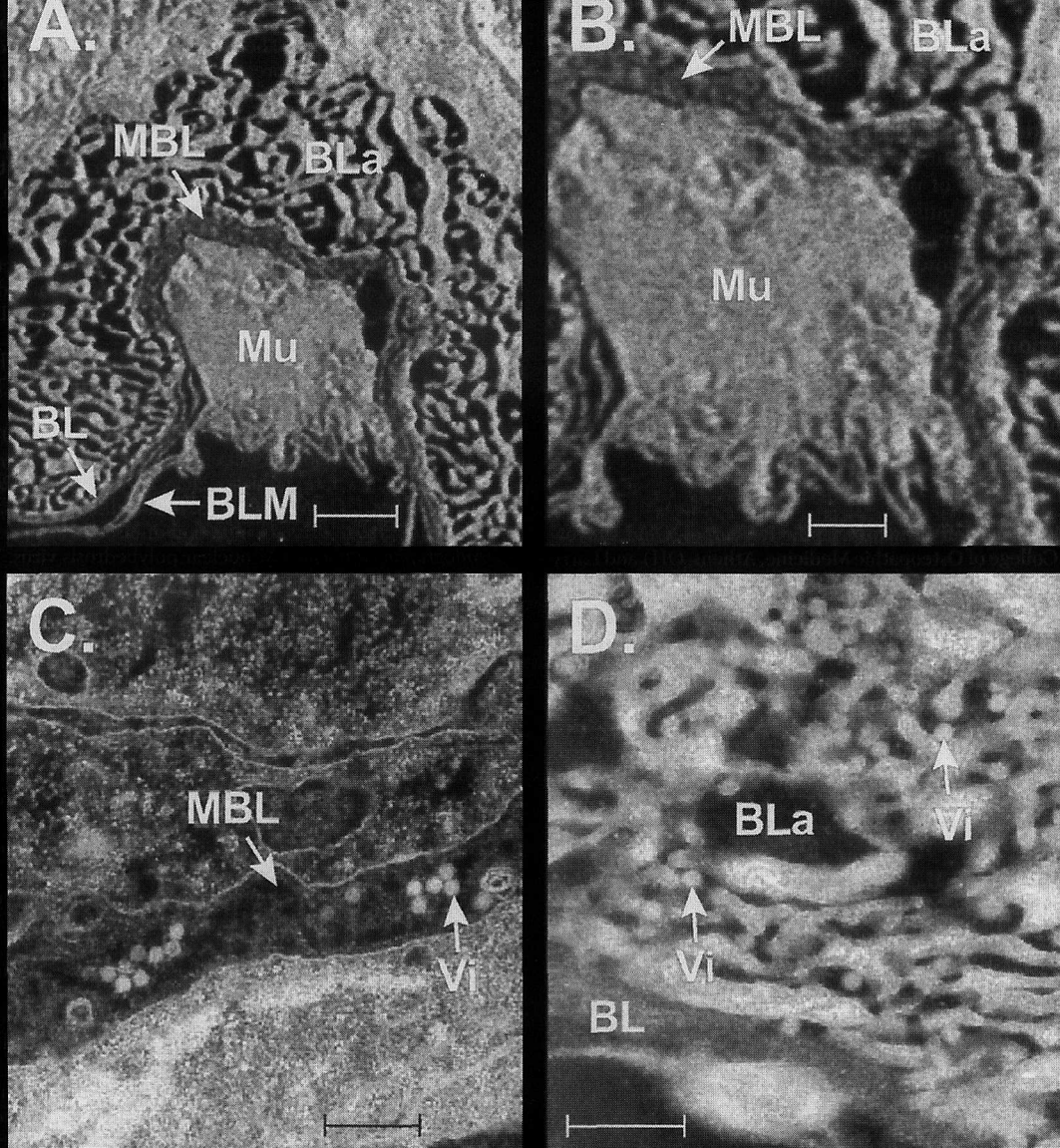


Fig. 5. Sections of the midgut in *Cx. pipiens*. (A) modified basal lamina associated with muscle (scale bar, 1 μ m). (B) Magnified portion of A (scale bar, 0.5 μ m). (C) Rift Valley fever virions in modified basal lamina (matrix) of proventriculus (scale bar, 0.5 μ m). (D) RVF virions budding into the basal labyrinth of midgut epithelial cells (scale bar, 0.5 μ m). BL, basal lamina of midgut; BLa, basal labyrinth; BLM, basal lamina of muscle; MBL, modified basal lamina; Mu, muscle; Vi, virion.

extrinsic incubation period, which is the time interval between the ingestion of a viremic blood meal and the ability of a mosquito to transmit a pathogen (Hardy 1988). For example, high viral doses would be expected to increase the probability that a midgut epithelial cell close to a tracheal cell or visceral muscle would become infected with resultant rapid dissemination and subsequent transmission. Cases of midgut infection without dissemination, that is dissemination "barriers" (Hardy 1988), could be explained on the basis that virus failed to reach a conduit within the chosen period of incubation in the laboratory, or if it

did reach a conduit, failure to infect due to the absence, low density, or inaccessibility of appropriate viral receptors in cells of the conduit system.

In view of the proposed routes of viral egress from the midgut epithelium, the use of leg assay to determine whether an arbovirus has disseminated from the midgut (Turell et al. 1984) should be viewed cautiously. Virus could exit from the midgut but remain within a portion of the tracheal system and closely associated tissues. Thus, the absence of virus in dissected legs would not necessarily indicate a non-disseminated infection.

In conclusion, the results of our VEE-VRP study are consistent with the operation of a viral dissemination conduit that involves tracheae and midgut muscles and the action of unmodified basal lamina as a barrier to arbovirus passage. Consistent with these results, histological and ultrastructural studies of *Cx. pipiens* provide evidence of tracheal cells in direct plasma membrane contact with midgut epithelial cells, and the presence of modified basal lamina in association with visceral muscle that closely resembles the modified basal lamina found in the proventriculus and that is known to allow passage of RVF virus. Together, and with consideration of other results in the literature, our findings contribute to answering a longstanding question in arbovirology: the mechanism by which arboviruses disseminate from the midgut in their mosquito hosts, thereby facilitating transmission to humans and other animals.

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